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Diffuse noxious inhibitory controls and brain networks are modulated in a testosterone-dependent manner in Sprague Dawley rats

Joyce T. Da Silva*, Youping Zhang, Jamila Asgar, Jin Y. Ro, David A. Seminowicz

Department of Neural and Pain Sciences, School of Dentistry, Center to Advance Chronic Pain Research, University of Maryland, Baltimore, United States

Abstract

Diffuse noxious inhibitory control (DNIC), which involves endogenous pain modulation, has been investigated as a potential mechanism for the differences in pain modulation observed between men and women, though the literature shows contradictory findings. We used a capsaicin-induced DNIC behavioral assay and resting state functional magnetic resonance imaging (rsfMRI) to assess the effect of testosterone on pain modulation and related brain circuitry in rats. We hypothesized that testosterone is required for DNIC that leads to efficient pain inhibition by increasing descending pain modulation. Male, female, and orchidectomized (GDX) male rats had a capsaicin injection into the forepaw to induce DNIC and mechanical thresholds were observed on the hindpaw. rsfMRI scans were acquired before and after capsaicin injection to analyze the effects of DNIC on periaqueductal gray (PAG), anterior cingulate cortex (ACC) and nucleus accumbens (NAc) connectivity to the whole brain. The strength of DNIC was higher in males compared to females and GDX males. PAG connectivity with prelimbic cortex (PrL), ACC and insula was stronger in males compared to females and GDX males, whereas females and GDX males had increased connectivity between the right ACC, hippocampus and thalamus. GDX males also showed a stronger connectivity between right ACC and NAc, and right NAc with PrL, ACC, insula and thalamus. Our findings suggest that testosterone plays a key role in reinforcing the endogenous pain inhibitory system, while circuitries related to reward and emotion are more strongly recruited in the absence of testosterone.

Keywords

Diffuse noxious inhibitory control; Testosterone; Brain networks; Periaqueductal gray; Anterior cingulate cortex; Nucleus accumbens

1. Introduction

Several recent human and animal studies have reported the importance of sex differences in pain perception and modulation in both acute and chronic pain states [1–7]. Multiple

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*Corresponding author at: Department of Neural and Pain Sciences, School of Dentistry, University of Maryland, Room 8404, Baltimore, United States. jteixeira@umaryland.edu (J.T. Da Silva).

physiological systems, including the endocrine system through estrogens, progestins and androgens, may affect the experience of pain directly or indirectly [1,8]. Recently, we have demonstrated that testosterone regulates μ -opioid receptor and cannabinoid 1 receptor (CB1) expression via transcriptional activities of androgen receptor in a trigeminal pain model [9,10]. These findings add novel perspectives on how male gonadal hormones modulate pain responses and have important clinical implications [6,10] since chronic pain conditions are more prevalent in women [11].

Testosterone has been shown to have analgesic effects in several preclinical as well as in clinical pain models [3,6,8,12–15]. Depletion of testosterone by gonadectomy increases formalin-induced nociceptive responses [16] in male rats, while replacement of testosterone to physiological levels decreases nociception in the formalin test [17]. Furthermore, therapy based on testosterone deprivation is associated with increased levels of pro-inflammatory factors and decreased levels of anti-inflammatory cytokines [18,19], while testosterone supplementation reduces inflammatory markers in both young and old hypogonadal men [18]. These analgesic events require activation of several brain circuits [20,21], but the role of testosterone in affecting brain networks in pain have been scarcely investigated.

One of the mechanisms that contribute to generally greater prevalence of pain in females than males is the sexually dimorphic activity in endogenous pain inhibitory systems. Diffuse noxious inhibitory controls (DNIC) require a noxious conditioning stimulus to one part of the body, which inhibits pain in other body regions [22–25]. While previous human studies indicated that DNIC effects were greater in males than females [26,27] sex differences in DNIC in animal models of pain have not been demonstrated. DNIC is mediated by a supraspinal mechanism, partly opioid-dependent [28], that modulates pain processing by the periaqueductal gray (PAG), locus coeruleus, and rostral ventromedial medulla (RVM) projections to the spinal cord [28,29]. DNIC increases activity in the orbitofrontal cortex (OFC) and amygdala, and reduces activations in primary and secondary somatosensory cortices (SI and SII), supplementary motor area (SMA), posterior insula and ACC [30]. However, the brain areas that are engaged in DNIC response by sex differences are still unknown.

Human brain imaging studies have shown sex differences in the brain areas involved in pain perception and modulation [31,32]. In irritable bowel syndrome patients, regional brain activity was greater in limbic areas, including the ventromedial prefrontal cortex, right ACC and left amygdala after visceral stimulus in female patients [33]. In contrast, male patients showed greater activation in brain regions involved in cognition and descending pain modulation, including the right dorsolateral prefrontal cortex, insula, and dorsal pons/PAG [33]. These findings suggest that females and males potentially differ in pain-related brain activation through different pathways associated with the multidimensional nature of pain [20,21]. However, very few studies have shown how testosterone in specific affects pain and brain activity [34,35]. Men with low levels of testosterone have greater activation in the pregenual ACC and OFC during thermal noxious stimulus, regions associated with pain-related unpleasantness [34]. Furthermore, while the activation of primary somatosensory cortex, a region associated with pain intensity, did not differ between groups, pain intensity ratings were higher in males with lower testosterone levels, compared to those with higher

levels [34]. Additionally, testosterone may be a key factor in modulating pain sensitivity via descending pathways, even in women. For example, on study reported increased RVM activity after thermal noxious stimulus was associated with higher testosterone levels in women using combined oral contraceptive pill [35].

In the current study, we assessed the effects of testosterone on endogenous pain modulation by examining DNIC responses and resting state fMRI in three groups of rats with clearly different circulating testosterone levels: young males, young females, and orchidectomized (GDX) male rats. We hypothesized that testosterone plays a key role in activating the endogenous pain modulatory systems by increasing connectivity of brain regions implicated in descending pain inhibition during DNIC.

2. Methods

2.1. Animals

Age-matched adult male, female, and GDX male Sprague-Dawley rats (8 weeks old; 250–300 g for males and 225–260 g for females; Harlan Laboratories Inc, Indianapolis, IN, USA) were used in the present study. GDX rats received the orchidectomy surgery at the time of order. They were used 3 weeks after the surgery. All other animals were also used 3 weeks following arrival to match the age and weight at the time of experiment. In order to confirm the efficacy of GDX surgery we assessed testosterone serum level from the GDX rats and compared to those obtained from intact male and female rats (The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core). Total free testosterone level in GDX rats (0.08 ± 0.03 ng/mL) was significantly lower than that of intact male rats (2.4 ± 0.4 ng/mL) and lower than that of female rats (0.29 ± 0.4 ng/mL) (Fig. 1), suggesting that the surgery completely depleted testosterone from GDX rats. The estrous cycle phase in female rats was not determined in this study. Animals were housed in a temperature-controlled room under a 12:12 light-dark cycle with access to food and water ad libitum. Rats in the same experimental groups were housed together as a pair. Male and female rats were housed in the same colony room and each experimental group was tested at a different time. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under a University of Maryland-approved Institutional Animal Care and Use Committee protocol. For DNIC behavioral testing, 6 rats were used for the intact male group, 7 rats for the GDX male group and 7 rats for the intact female group. The behavioral experiments using phosphate-buffered saline (PBS) as control had 5 rats per group. fMRI study investigated DNIC effects after capsaicin injection in males, GDX males and females from a separate group of rats consisting of 4 rats per group.

2.2. Behavioral assay

Our model of DNIC in healthy animals was adapted from an earlier study [36]. Hindpaw withdrawal thresholds to noxious mechanical stimulation were measured before and 15, 30, 45, 60 and 90 min following the administration of a small volume of capsaicin (1% in 30 μ l) into the left forepaw. The same volume of PBS was injected in the same manner in the control groups. Mechanical sensitivity of the left hindpaw was assessed with the

Randall–Selitto test, an established rodent model for testing mechanical hypersensitivity of the paw. Animals were first allowed to habituate to the experimental room for 30 min for three consecutive days. The withdraw response to noxious paw pressure was assessed using a digital paw pressure Randall–Selitto applicator for rodents (IITC Life Science, Woodland Hills, CA). Each rat was placed in a cloth holder suspended in a sling, and the probe of the pressure applicator was placed under the plantar surface of the hindpaw. The probe has a spring load for easy opening and closing of the pressure applicator. The probe closes the pressure applicator and captures the pressure upon reaction. A gradually increasing pressure is applied until the rat withdraws its hindpaw. The lowest pressure necessary to elicit the withdraw response prior to capsaicin treatment was considered as the baseline mechanical threshold. Results were analyzed using the statistical analysis software package SigmaPlot. Two-Way Repeated Measures ANOVA with Holm-Sidak method for correction of multiple comparisons were performed to determine significant group and time effects. Differences were considered statistically significant at $p < .05$ and the data were presented as mean \pm standard error of the mean (S.E.M.). The investigators conducting the behavioral study were blinded to the experimental group and drugs.

2.3. rsfMRI data acquisition

Proton density-weighted images were obtained using a 2D RARE (342×294 matrix, 24 coronal 1 mm slice thickness, in plane resolution $100 \mu\text{m}$, TR 2000 ms, TE 28 ms) for anatomic reference. rsfMRI scans were acquired using an echo planner imaging (EPI) sequence (TR 1500 ms, TE 37 ms, 75×63 matrix, in plane resolution $0.45 \times 0.45 \times 1$ mm, 24 coronal slices, 620 volumes per scan). The anatomical and the first rsfMRI scans were performed for each rat as baseline (prior to capsaicin injection, 15.5 min). Rats subsequently received an injection of capsaicin (1% in 30 μl) into the left hindpaw and a second rsfMRI scan was acquired for 15.5 min. Isoflurane concentrations were kept below 1.5% and maintained throughout the scan session. Respiration and heart rate, as well as body temperature were monitored continuously throughout the experiment. Data were acquired using a Bruker BioSpec 70/30USR Avance III 7-Tesla scanner (Bruker Biospin MRI GmbH, Germany) and a 40-mm circular polarized volume coil. The investigators running the MRI session were blinded to the experimental group.

2.4. rsfMRI preprocessing and analysis

All preprocessing and analyses were performed in SPM12 (<http://www.fil.ion.ucl.ac.uk/spm/>). We used seed-based analysis to assess how connectivity to periaqueductal gray (PAG), right anterior cingulate cortex (R ACC) and right nucleus accumbens (R NAc) varies after capsaicin injection between the different groups. PAG was selected based upon prior literature showing activation during descending pain modulation [20,37]. ACC is involved in the emotional aspect of pain and sends projections to the PAG [20,38]. NAc has connections with ACC and participates within the reward/motivation network in pain state [21,39]. For lateralized regions, the side contralateral to capsaicin injection was used. Seeds were chosen based on anatomical locations in the Paxinos and Watson atlas (2004). We first created an anatomical template of T2-weighted images [40]. Functional data underwent the following preprocessing steps: slice timing correction, motion correction, normalization to the group average template, which included interpolation to a

voxel size of 0.5 mm isotropic, bandpass filtering(0.009–0.2 Hz), and smoothing at 1 mm FWHM. We then extracted time series data from the regions of interest and regressed these time-courses with the signal at each voxel across the whole brain to reveal connectivity patterns for each animal. Six motion parameters were included as regressors of no interest. Second level analyses included two sample *t*-test to evaluate group differences from male versus female, female versus GDX, and male versus GDX after capsaicin injection. Because of the small sample size and exploratory nature of the study, second level maps used cluster-forming (voxel level) thresholds at $p < .05$, $.005$, and $.001$ uncorrected and significant clusters ($p < .05$) for each threshold are reported. We note that the cluster-forming threshold of $p < .05$ is overly liberal but we present these results as exploratory. For visualization, we extracted and plotted the averaged adjusted beta values from significant clusters for each animal and time-point.

3. Results

3.1. The analgesic effect of DNIC is attenuated in female and GDX male rats

Studies have shown previously that the analgesia produced by DNIC is observed in response to remote noxious stimulus in rats [29,41]. In this study, DNIC was induced by capsaicin injection into the forepaw and mechanical thresholds were assessed on the hindpaw. Main effects of group ($F = 11.5$, $p < .001$), time ($F = 108.1$, $p < .001$) and group \times time ($F = 5.1$, $p < .001$) were statistically significant in the behavioral data (Fig. 2A). Post-hoc analysis indicated a statistically significant increase of DNIC in intact males at 15, 30 and 45 min post capsaicin injection compared to females ($p < .025$) and GDX male rats ($p < .001$) (Fig. 2A). GDX males and females exhibited similar extent and duration of DNIC responses. Significant increase in hindpaw mechanical threshold, indicating analgesia, was observed 15, 30, and 45 min after capsaicin injection into the forepaw in male rats compared to the baseline mechanical threshold (Pre) ($p < .001$). The increase in mechanical threshold gradually subsided to the baseline level within 60 min. Female and GDX male rats also showed the significant analgesic effect, which lasted for 30 min after capsaicin injection compared to the Pre ($p < .001$). DNIC responses were not observed after PBS injection into the forepaw in any of the groups (Fig. 2B). These findings supported our hypothesis that males exhibit more efficient DNIC response and that DNIC is modulated in a testosterone-dependent manner.

3.2. Testosterone-dependent changes in functional connectivity after DNIC induction

To explore the role of testosterone in whole brain connectivity, we used PAG, R ACC and R NAc as seed regions and capsaicin injection as noxious stimulus to induce DNIC in the three groups of rats.

For PAG seed, connectivity in males was significantly increased with PrL, ACC and insula, compared to females and GDX males after capsaicin injection (Fig. 3). Since we had observed significant differences in PAG connections with ACC in males compared to females and GDX males, we further investigated the R ACC connectivity with the whole brain. We found that in females and GDX males, R ACC had increased connectivity with hippocampus and thalamus in one cluster, compared to the male group (Fig. 4). In addition,

R ACC showed stronger connectivity with NAc, insula and amygdala in GDX males, relative to female and male rats (Fig. 5). Our data demonstrate that GDX males and females had similarities in connectivity of brain areas during DNIC induction compared to males, such as hippocampus and thalamus.

GDX males also showed a stronger connectivity between R ACC and NAc compared to males, leading us to investigate the NAc connections in this study (Fig. 6). Increased connectivity of the R NAc to the PrL, ACC, insula, and thalamus was evident in GDX males, while males showed decreased connectivity and females did not show any change after capsaicin injection in these same brain areas (cluster-forming $p < .005$ – Fig. 6). GDX condition appears to alter the neuronal pathways recruited during DNIC such that the brain areas related to emotion and reward [42] are strongly connected in the complete absence of testosterone.

4. Discussion

This is the first study to demonstrate sex differences in DNIC in an animal model. Animal models that exhibit clear sex differences in DNIC would allow back-translation of clinical or human psychophysical observations and provide important tools to investigate mechanisms underlying sex differences. The primary finding of this study was more efficient DNIC responses observed in males compared to females and GDX males. Human studies suggest that women are more pain sensitive and process less efficient DNIC compared to men in both healthy and pain states [43–45]. Our data further suggested that testosterone plays an important role in modulating DNIC response promoting higher analgesia in males since the absence of testosterone in the age and weight matched GDX males led to DNIC responses similar to those of females.

The analgesic responses to capsaicin we observed could also be interpreted as stress-induced analgesia (SIA) since there are remarkable overlap between the mechanisms mediating DNIC and SIA. Recent studies have shown that both spinal and supraspinal pathways underlie both phenomena and that both phenomena involve opioidergic as well as cannabinoidergic modulations [46–48]. However, capsaicin as a stressor to induce SIA has actually been only scarcely reported in the pain literature. Gear et al. showed that capsaicin-induced analgesia was unaffected with stress axis lesions (i.e., either hypophysectomized or adrenalectomized) [36]. More importantly, plasma corticosterone levels in intact animals did not change when measured before capsaicin injection or after the onset of antinociception [36]. Suplita et al. showed that the blockade of TRPV1 receptors with capsazepine, in a model of intraperitoneal capsaicin, did not alter cannabinoid SIA, suggesting that cannabinoid SIA was not modulated by capsaicin as a stressor [49]. These studies suggest that an acute capsaicin treatment does not lead to effective stress responses required for SIA. It is well established that capsaicin is a potent noxious stimulus that lead to profound pain responses when administered into human subjects. Thus, the capsaicin-induced analgesia in our report is more likely to involve the concept of “pain inhibits pain”, which describes DNIC phenomenon.

Another principal finding in this study is that there is a remarkable correlation between the behavioral data and fMRI data. While it is possible that our imaging data may reflect the animal responses to capsaicin's acute effect on pain we suggest that the brain responses we observed might reflect DNIC for the following reasons: In our study, we injected a low dose of capsaicin (0.3 μg) into the hindpaw and the fMRI scan was acquired during 15.5 min after injection. We assumed that the acute pain effect from the capsaicin injection will be most prominent during the first 5 min of the fMRI scan since previous studies showed that acute pain evoked by capsaicin generally lasts 5 min in rats [50]. Jantsch et al. in 2009 also showed that capsaicin injected intradermally in humans resulted in a strong burning pain that decays exponentially within 2 min (doses of 0.05, 1 and 20 μg) [51]. Several studies have been using capsaicin to induce acute pain, however they apply higher doses and different routes of administration [52]. Therefore, it is possible that we are capturing both acute pain and DNIC effects of capsaicin with the time frame we employed in this study. Interestingly, the fMRI findings were supported by the strong correlation with the behavioral data showing the testosterone effects on DNIC.

We and others have shown that the analgesic effect of testosterone is promoted, at least in part, by the μ -opioid receptor regulation [9,53], and PAG has been shown to be a key neural locus for sex differences in opioid action [5,53–55]. Thus, we first examined PAG connectivity with the whole brain as a potential mechanism mediating the testosterone effect on DNIC. Males showed increased functional connectivity of the PAG seed to PrL, ACC and insula, compared to females and GDX males. This finding suggests that more efficient DNIC in males is mediated by a stronger PAG and cortical connections. Previous reports indicating that men display a pain-induced increase in PAG functional connectivity that is not observed in women is consistent with our data [56,57]. A similar PAG connectivity between female and GDX male rats further suggests that testosterone may play an essential role in recruiting endogenous pain modulation circuitry during DNIC. Literature indicates that ACC exerts top-down influences on the PAG to gate pain modulation during analgesic strategies and opioidergic signaling is critical for this network [58–61]. Therefore, we also investigated the ACC connectivity. Our data showed higher functional connection of ACC with hippocampus and thalamus in females and GDX males compared to males, further clarifying the sex differences on recruited pathways during DNIC. Accordingly, studies have shown that hippocampus responds to external agents such as hormones, stressors and pain [62,63]. Estradiol can increase NMDA binding and spinogenesis in the CA1 region of females, which may contribute to sex differences in mood, pain and cognitive function [63]. Taken together, our data indicate that during DNIC, testosterone facilitates the inhibitory exertion of PAG onto ACC, and that the lack of testosterone attenuates this connectivity, resulting in increased connectivity of ACC to hippocampus and thalamus in females and GDX males.

Another novel finding was that GDX males had stronger connectivity of the ACC seed to NAc, insula and amygdala, compared to females and males. The NAc receives afferent nociceptive information through connections with the thalamus, parabrachial area, amygdala and ACC [64,65]. The dopaminergic neurotransmission in the NAc does not seem to encode pain sensory information, but rather signals affective value and saliency of the stimulus [66]. Pain onset is considered aversive and its offset represents a potential reward, thus

the brain reward circuitry represents a vital element for pain experience and modulation [67]. Consequently, NAc has emerged as a crucial structure in this circuitry [68]. We also observed an increase in connectivity between the NAc seed to PrL, ACC, thalamus and hippocampus in the GDX male group. The brain reward/motivational mesocorticolimbic circuitry depends on opioidergic circuits in the ACC and downstream dopaminergic signaling in the NAc. In addition, stress and anxiety circuitry shows connections between ACC and insula, as well as projections to NAc that receives glutamatergic outputs from the amygdala and dopaminergic terminals from the VTA [64,65]. GDX males may recruit different neuronal pathways during DNIC compared to males, since areas related to reward and emotion are strongly connected in the absence of testosterone. Together, these findings may contribute to the understanding of the testosterone modulation in brain connectivity and analgesia.

There are certain limitations to this exploratory study. First, the use of isoflurane as anesthesia in the rsfMRI protocol. We used isoflurane because the resting state networks are generally preserved under sedation and highly reproducible between animals, compared to the awake condition [69]. The movement and stress effects in awake animals are still a concern in the neuroimaging community [70–72]. Second, the capsaicin injection induced the DNIC response, although there is also an acute pain effect from the same stimulus. Future studies could explore whether the changes in functional connectivity observed in this study result from the DNIC or acute pain induced by the same stimulus. Third, the capsaicin injection was performed in the forepaw for behavioral assay and in the hindpaw for rsfMRI protocol due to the inaccessibility of the forepaw during the imaging scan. Fourth, we report rsfMRI results at 3 cluster forming thresholds, the highest being $p < .05$, which is typically considered overly liberal and prone to false positives [73]. Future work with larger sample sizes would be required to show reliability of the findings. Further investigation including more control groups to investigate opioid and dopaminergic receptors, pharmacological interventions and behavioral tests is needed to expand the mechanisms involved in sex-hormone related modulation of brain networks during DNIC.

5. Conclusions

The major aim of the current study was to evaluate the testosterone effect on DNIC response and resting state networks. We observed a modulation in brain connectivity by DNIC induction in a sex-dependent manner. Although this is an exploratory study, the higher analgesic response and PAG connectivity in males indicate a stronger participation of DNIC in the presence of testosterone. Nonetheless, females recruited more hippocampus and thalamus under the same DNIC induction. GDX also showed a particular connectivity of circuitries related to reward and emotion, which could be enforced by the absence of testosterone. This study has an important clinical and research implication, since we show for the first time that DNIC response and brain connectivity can be influenced by testosterone. Our study should provide important neurobiological basis for enhancing our understanding on sex differences in chronic pain conditions, and the development of mechanism-based therapeutic approaches customized for men and women.

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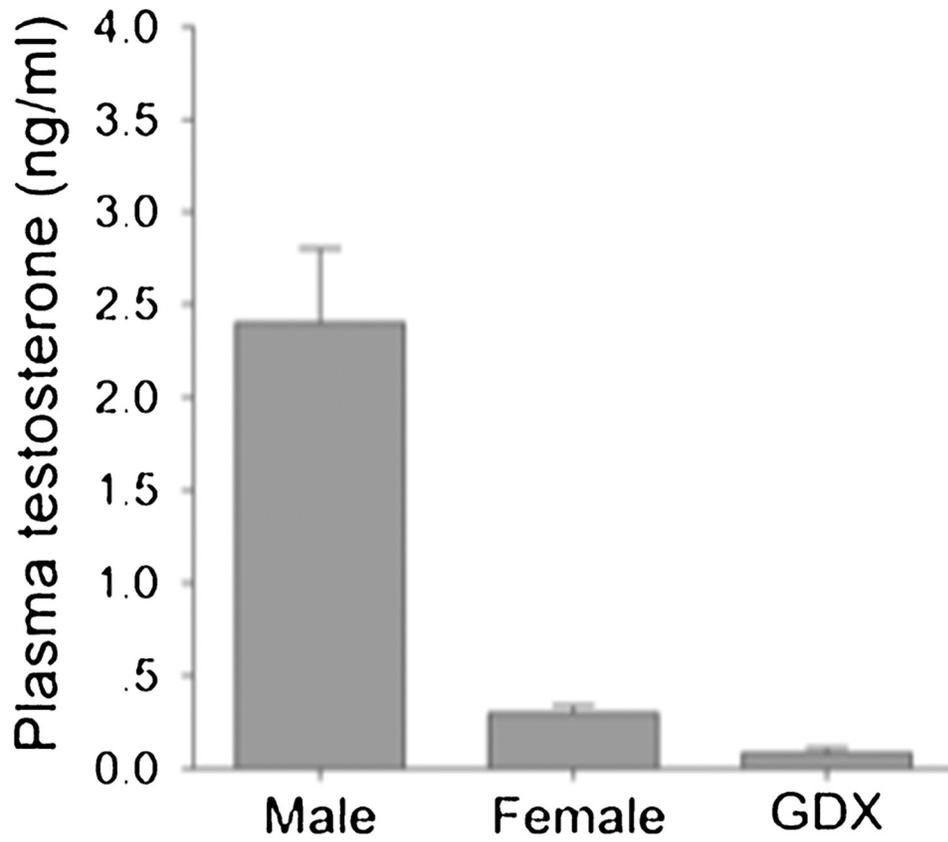


Fig. 1. Blood samples were obtained from the tail vein of normal intact male, female and GDX male rats. Total free serum testosterone levels (ng/mL) from the blood samples were assessed by ELISA assay kits by IBL.

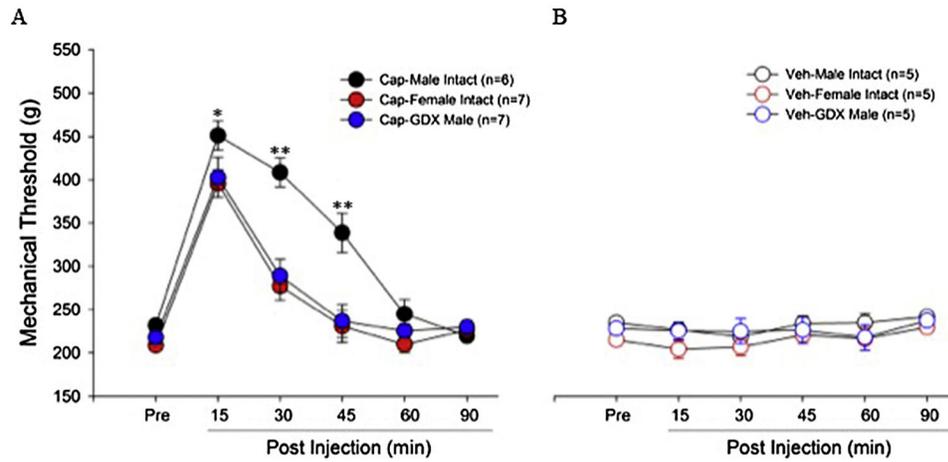


Fig. 2.

Testosterone significantly increases DNIC. Forepaw injection of Capsaicin (A), but not PBS (B), significantly increased mechanical thresholds (i.e., DNIC) of the hindpaw in intact male and female, and GDX male rats. (A) Males had significantly more DNIC than females and GDX males (* $p < .025$ and ** $p < .001$). Data are mean \pm S.E.M., two-way ANOVA with Holm-Sidak method.

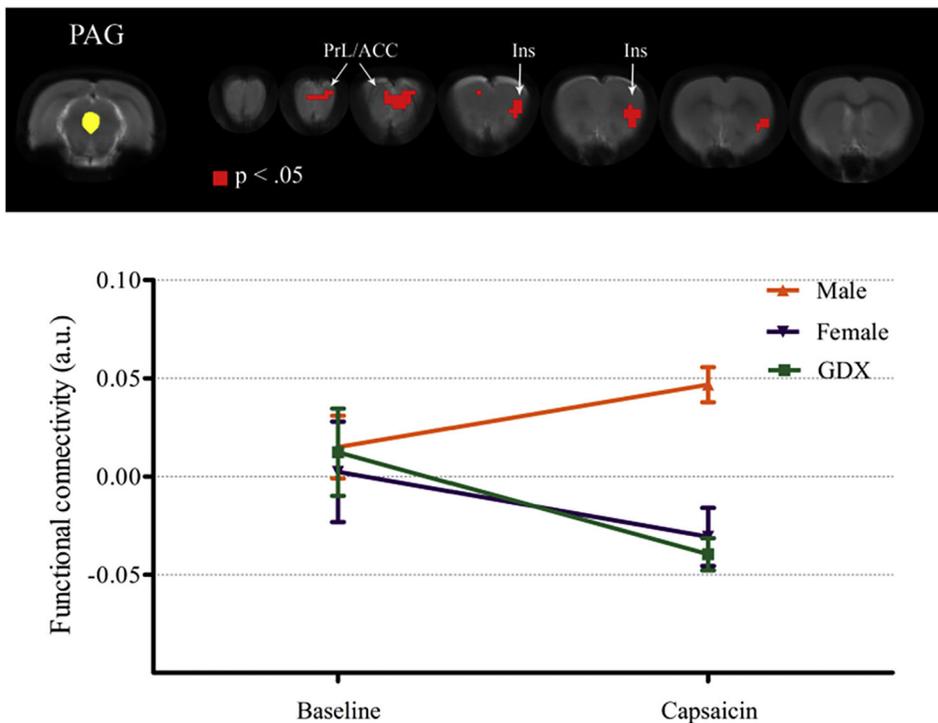


Fig. 3. PAG seed region (yellow) and cluster with stronger connectivity with PrL, ACC and insula (red) in males, compared to females and GDX males. Plot shows extracted beta values (a.u.) from significant clusters (average \pm S.E.M and $p < .05$) with cluster-forming thresholds at $p < .05$, $.005$, and $.001$ for each group and time-point. PrL: prelimbic cortex, ACC: anterior cingulate cortex, Ins: insula and a.u.: arbitrary units (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

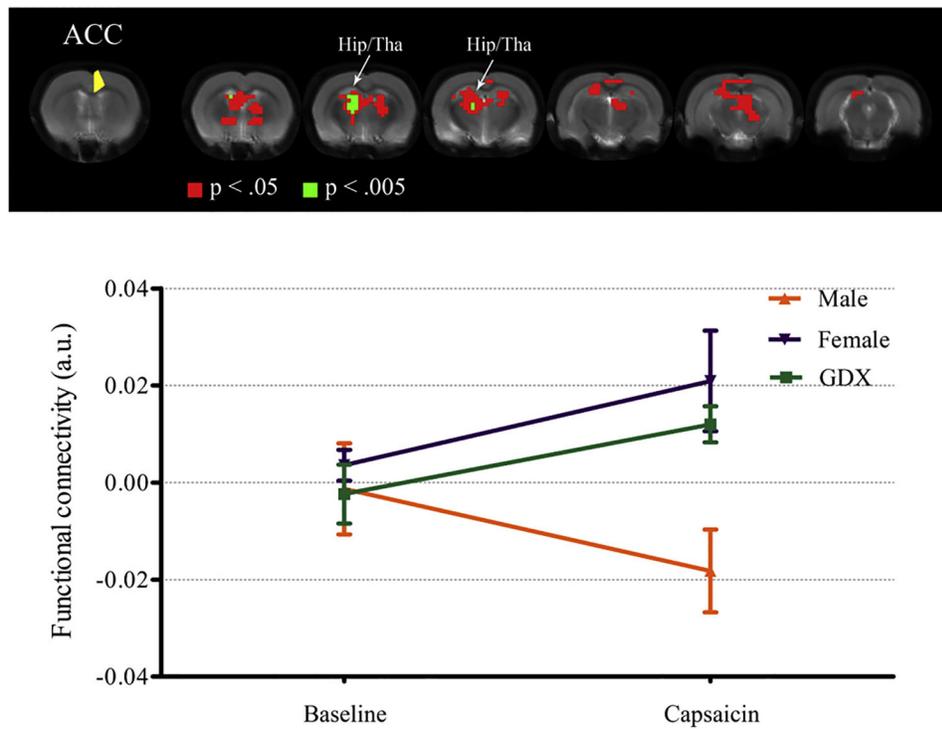


Fig. 4. Right ACC seed region (yellow) with stronger connectivity with hippocampus and thalamus in females, compared to males and GDX males. Plot shows extracted beta values (a.u.) from significant clusters (average \pm S.E.M and $p < .05$) with cluster-forming thresholds at $p < .05$, $.005$, and $.001$ for each group and time-point. Hip: hippocampus, Tha: thalamus and a.u.: arbitrary units (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

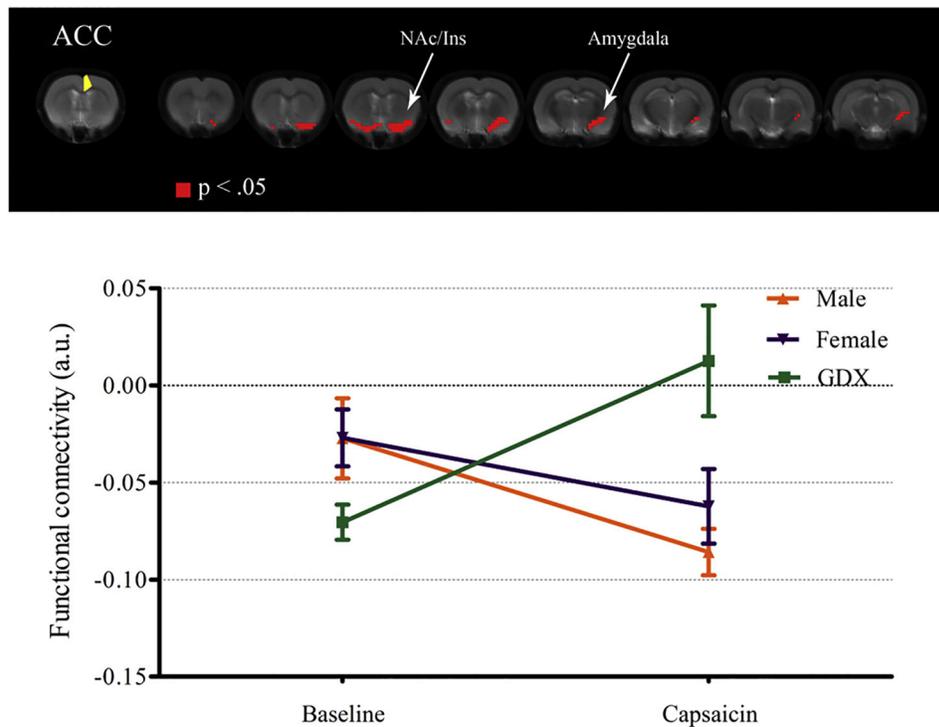


Fig. 5. Right ACC seed region (yellow) and cluster with stronger connectivity with NAc, insula and amygdala (red) in GDX males, compared to females and males. Plot shows extracted beta values (a.u.) from significant clusters (average \pm S.E.M and $p < .05$) with cluster-forming thresholds at $p < .05$, $.005$, and $.001$ for each group and time-point. NAc: nucleus accumbens, Ins: insula and a.u.: arbitrary units (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

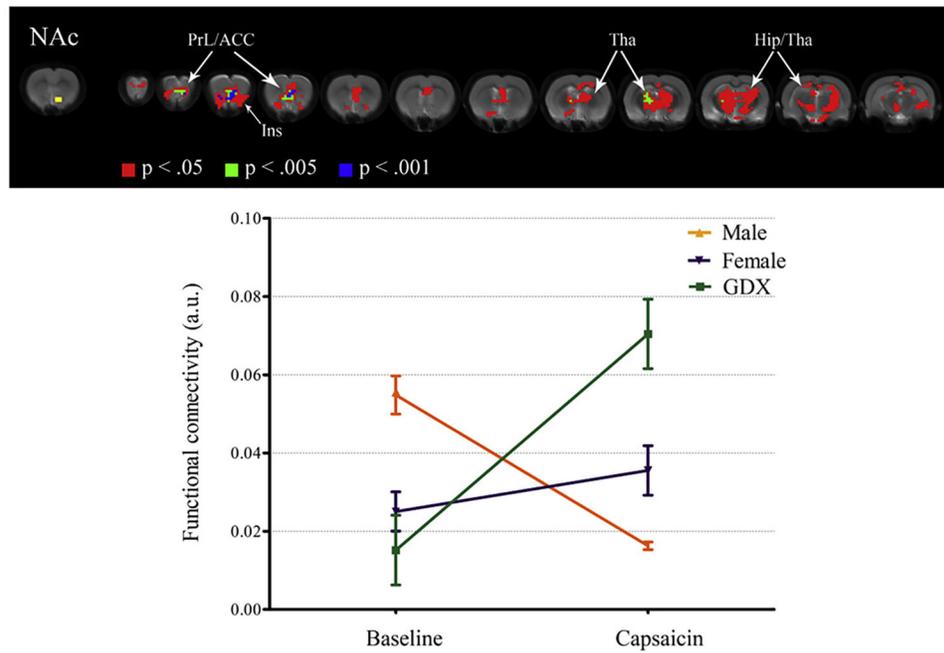


Fig. 6. Right NAc seed region (yellow) with stronger connectivity with PrL, ACC, insula, thalamus and hippocampus in GDX males, compared to females and males. In contrast, male had decreased connectivity in the same areas after capsaicin injection. Plot shows extracted beta values (a.u.) from significant clusters (average \pm S.E.M and $p < .005$) with cluster-forming thresholds at $p < .05$, $.005$, and $.001$ for each group and time-point. PrL: prelimbic cortex, ACC: anterior cingulate cortex, Ins: insula, Tha: thalamus, Hip: hippocampus and a.u.: arbitrary units (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).